JOURNAL OF BACTERIOLOGY, June 1971, p. 949-954
Vol. 106, No. 3

Printed in U.S.A.

Variation in the Primary Structure of *Bacillus subtilis* Flagellins[1]

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Received for publication 4 March 1971

The flagella derived from 18 strains *Bacillus subtilis* were tested for their reaction with antiflagellar filament antibody and antiflagellin antibody. On the basis of their reactivity, at least five serologically distinct classes could be identified. Peptide map analysis of tryptic digests of the subunit proteins were consistent with the immunochemical analysis. Large differences in sequence existed among proteins of the different classes; proteins within an antigenic group differed by only a few peptides. Furthermore, 9 of the 27 tryptic peptides resolved were common to flagellin proteins from all the classes examined. The relationship between antigenic specificity, variability in peptide pattern, and the conformation of the flagellin protein are discussed.

An extensive analysis of the antigenic components of *Salmonella* (8) flagella has demonstrated that a high level of immunological variation exists among the flagella of related strains. By contrast, no thorough comparative analysis of *Bacillus subtilis* flagella has been reported, even though such analysis might provide a simple means of distinguishing *B. subtilis* and related strains. We have prepared specific antisera against flagellar filaments derived from various strains of *B. subtilis* and against the monomeric protein, flagellin, which comprises the filament. These antisera were used to arrange strains of *B. subtilis* into discrete groups having specific antigenic characteristics. The basis for the immunological variation was further explored by comparing tryptic peptide maps of flagellin from the different serological classes.

MATERIALS AND METHODS

Bacterial strains. Most of the strains of *B. subtilis* were from the American Type Culture Collection; they are referred to by their ATCC number. BD71, a 168 Marburg derivative, was obtained from D. Dubnau, and W23 was obtained from J. Spizizen.

Media. Bacteria were grown in a minimal salts medium (14) supplemented with 0.1% Casamino Acids and 15 μg of any known required nutrients per ml. Cultures were normally incubated at 37 C.

Preparation of antigens and antibodies. Flagella were prepared as previously described (2). They were removed from the bacteria by shearing, concentrated by centrifugation, and finally purified by ion-exchange chromatography (10) or by differential centrifugation. The flagellar filaments were disaggregated to produce flagellin by adjusting the *p*H to 2.4 with hydrochloric acid and incubating at room temperature for 10 min. The solution was then centrifuged at 100,000 $\times$ *g* for 1 hr. The supernatant fluid was adjusted to the desired *p*H by the addition of KOH or NaOH. All of the flagellin preparations gave a single band on acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Antiflagellar filament antibody was prepared by intravenous injection of the flagellar filaments. Intravenous injection of flagellin subunits resulted in antisera with high titers against both the subunit and the filament. The antifilament antibody might have resulted from a small amount of flagellin that aggregated before injection. To prevent any aggregation and to obtain a more specific high titer antiserum, freshly prepared flagellin was first bound to methylated albumin and then injected. BD71 flagellin was mixed with an equal weight of methylated serum albumin in 0.05 M tris(hydroxymethyl)aminomethane, *p*H 8.0, and 0.1 M NaCl. This was then mixed with an equal volume of incomplete Freunds Adjuvant (Difco) and injected into a white New Zealand rabbit in the toe pads, foot pads and intramuscularly. The initial injection contained 2 mg of flagellin. Three weeks later, another 2 mg of flagellin mixed with methylated albumin was injected, and one week later the animal was bled. Of three rabbits injected in this way, one responded with an antiserum that did not show significant complement fixation with flagellar filaments. This (Ra-65) was the antiflagellin used in subsequent work.

Measurement of antigen-antibody reaction. Two methods were used to quantitate the extent of reactions with the antibody, (i) the antibody-binding technique

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previously described (3), and (ii) complement fixation as described by Wasserman and Levine (15). The degree of cross-reaction in the complement-fixation system was expressed as the index of dissimilarity (13), i.e., the ratio of the antiserum concentrations used with the heterologous and homologous antigens to give the same amount of complement fixation.

Slide agglutination tests could be used to a limited extent with some of the strains tested. However, many of the strains grew as long chains or tended to clump during growth, and careful quantitative comparisons were difficult.

Tryptic peptide mapping. Flagellin was first treated with performic acid to eliminate artifacts arising from oxidation of methionine during the drying periods. The protein was oxidized at 0 C for 2.5 hr by the method of Hirs (5). Oxidized, lyophilized flagellin was then dissolved in 0.2 M NH_4HCO_3 (*p*H 8.7) at concentrations of 1 mg/0.15 ml of buffer. Trypsin (Worthington Biochemical Corp.; diisopropylphosphofluoridate treated) was added to a 1% (w/w) solution of flagellin, and the sample was incubated at 37 C for 2.5 hr, after which 1% more trypsin was added and the incubation was continued for an additional 8 hr. The digest was lyophilized and stored at −15 C or was diluted in 0.2 M NH_4HCO_3 buffer and applied to a cellulose thin-layer chromatogram. Approximately 300 μg of each protein was applied to the chromatogram. Electrophoresis was for 2.5 hr at 300 v, with pyridine-acetate at *p*H 5.2 used as buffer (2% pyridine, 1% acetic acid). The chromatograms were air dried overnight before chromatography in *n*-butanol-acetic acid-water (4:1:5) at right angles to the direction of electrophoresis. The chromatography step was repeated once to increase the resolution. Chromatograms were air dried and then dipped in the cadmium acetate-ninhydrin stain of Heilman et al. (4). The color developed during overnight incubation at room temperature.

To compare peptide maps, the digest was run alone, and it was also mixed with a digest of the BD71 or W23 prototype. Comparison of the individual maps with that of the mixtures provided a sensitive method for demonstrating small differences.

RESULTS

Specificity of antiflagella and antiflagellin sera. Figure 1B shows the reaction of antiflagellar antibody with flagellar filaments. At a dilution of 1/10,000 the antibody reacts with the filament but does not show direct complement fixation with flagellin subunits. On the other hand, the antiserum prepared against the subunits reacts strongly with the subunit protein but shows no reaction with flagellar filament (Fig. 1A). To further explore the specificity of these antisera, the heterologous antigens were tested for their ability to inhibit the homologous reaction. Flagellin is an effective inhibitor of the filament-antifilament reaction (Fig. 2). On the other hand, filaments have no effect on the reaction between subunits and antisubunit antibody. We can, therefore, tentatively conclude that the antisubunit serum is highly specific for antigenic determinants that are revealed only in flagellin, whereas antibodies prepared against flagellar filaments can also bind the flagellin, but the reaction with flagellin is not extensive enough (3) to lead to complement fixation.

Cross-reaction with antiflagellar antibodies. Antisera were prepared against the flagellar filaments isolated from four strains of *B. subtilis* which appeared to be antigenically distinct in preliminary qualitative agglutination tests. The degree of cross-reaction between the four antisera and flagellar filaments from 18 strains of *B. subtilis* was measured by the radio-immune assay of Grant and Simon (3). The strains tested define a minimum of five discrete immunological groups (Table 1); flagella from any one group apparently do not show marked cross-reaction with antisera prepared against flagellar filament of any other group. These initial results were confirmed by the complement-fixation technique which also quantitatively measured antibody-antigen complexes. No cross-reaction was detected between members of the discrete groups such as BD71 or W23. Increasing the concentration of antiserum to BD71 flagellar filaments to 10 times that required for 50% complement fixation with the homologous flagella did not produce a detectable cross-reaction with W23,13542, or 9799 flagella. Furthermore, antiserum to W23 flagella did not cross-react with BD71 flagella when concentrated eightfold above the titer necessary for 50% complement fixation with W23. Therefore, there are clearly distinct antigenic classes with very little cross-reaction between them.

Cross-reaction with antiflagellin antibodies. The degree of cross-reaction of seven different flagellins with specific antiflagellin antiserum was measured by complement fixation. The extent of cross-reaction was defined by the index of dissimilarity; an index of one corresponds to complete homology whereas an index greater than one indicates differences. The index for flagellin subunits (Table 2) ranged from 1.0 to 2.75. Flagellins that were in the same group (Table 1), e.g., BD71 and 9524 or W23 and 12695, had almost identical indices, whereas members of different groups could be distinguished from BD71.

However, in contrast to the pattern observed with flagellar filaments, all of the flagellins exhibited significant amounts of cross-reaction. For example, the reaction of anti-BD71 flagellin serum with W23 flagellin has an index of 1.8; in contrast, the reaction of anti-BD71 flagellar filament serum with W23 filament had an index greater than 10. Thus, whereas the flagellar filaments of different strains of *B. subtilis* could be

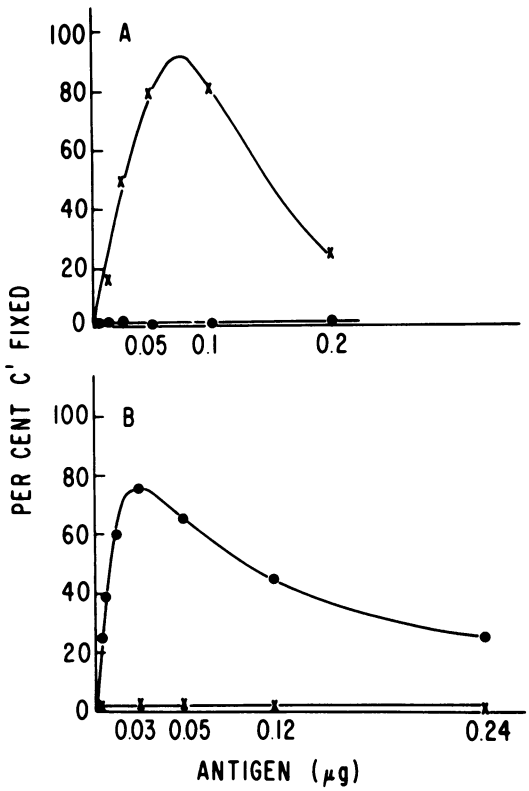

FIG. 1. *Immunochemical reaction of flagella and flagellin. (A) The complement-fixation reaction of anti-flagellin antibody (Ra 65), diluted 1:2,000, with flagellin (×) and flagella (●). (B) The reaction of anti-BD71 flagellar filament antibody diluted 1/10,000 with flagellar filaments (●) and flagellin (×).*

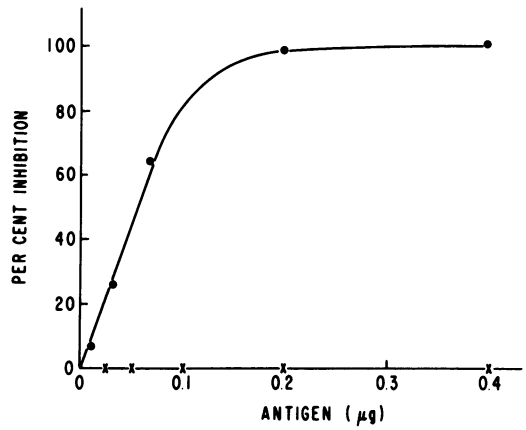

FIG. 2. *Inhibition of the homologous antifilament and antisubunit reactions with subunits and filaments. Anti-BD71, diluted 1:10,000, reacting with 0.03 μg of flagella in the presence of various amounts of flagellin (●). Antiflagellin antibody (Ra 65), diluted 1:2,000, reacting with 0.1 μg of flagellin in the presence of various concentrations of flagellar filament protein (×).*

assigned to a number of non-overlapping groups based on their reaction with antifilament antibody, the analysis of the cross-reaction with antisubunit antibody suggests that all of the flagellins share sufficient common antigenic determinants to give clearly measurable cross-reaction.

Fingerprints of flagellins. To further define the extent of homology and differences among the flagellins, tryptic peptide maps were compared.

Figure 3 shows a comparison of fingerprints of BD71 and W23. There are clearly a large number of peptide differences between these strains. The data from an extensive comparison are shown in Table 3. The 9524 and BD71 flagellin peptide maps were identical, in agreement with the high degree of antigenic cross-reactivity demonstrated with both antifilament and antisubunit sera. On the basis of the mapping data, 12695 flagellin differed from W23 by one or two tryptic peptides, supporting the observation that the two types of filaments as well as the flagellin

TABLE 1. *Antigenic comparison of flagellar filaments of Bacillus subtilis*[a]

Strain	Antiserum			
	BD71	W23	13542	9799
BD71	33,660	200	810	1,173
6051	35,189	233	2,589	3,894
3610	30,214	328	963	1,444
9524	28,512	382	374	2,210
4944	39,053	239	493	949
W23	916	17,782	905	1,058
10783	100	18,217	1,600	98
12695	833	12,157	120	932
13542	574	58	41,324	1,490
7480	975	363	35,607	35
9799	1,475	227	206	20,120
12100	156	235	927	23,236
8473	1,022	436	1,020	21,543
7067	922	368	723	946
14410	1,260	326	208	639
14415	909	328	709	1,201
14416	1,033	1,000	210	435
9858	839	147	742	1,751

[a] Cross-reactivity was measured by the radio-immune assay of Grant and Simon (3). The columns list the amounts of radioactive antibody bound (counts per minute background) to saturating levels of antigen. The nonspecific binding or background was 3000 counts/min with anti-BD71, 1,540 counts/min with anti-W23, 3,200 counts/min with anti-13542, and 1,820 counts/min with anti-9799.

TABLE 2. *Antigenic cross-reactivity of flagellins[a] of Bacillus subtilis*

Strain	Titer[b]	Index of dissimilarity
BD71	1:2,200	1.0
9524	1:2,200	1.0
12695	1:1,450	1.5
W23	1:1,200	1.8
13542	1:920	2.4
7067	1:880	2.5
9799	1:800	2.7

[a] Degree of cross-reaction with antibody to BD71 flagellin was measured by complement fixation.
[b] Dilution of anti-Ra65 that gives 50% reaction.

were antigenically similar. The 7067 flagellin was anomalous; although the peptide maps of 7067 and W23 had a superficial resemblance, 7067 flagella were antigenically distinct from W23 and BD71 flagellar filaments. Indeed, 7067 possessed peptides characteristic of both W23 and BD71 flagellins but nevertheless had lost the ability to cross-react with antisera to flagella filaments of either type. Both 9799 and 13542 peptide maps revealed new peptides absent in both W23 and BD71, as well as peptides common to all the flagellin examined. Thus, on the basis of detailed peptide comparisons, five classes of flagellin could be distinguished, and these classes were identical to those defined by the immunological studies.

DISCUSSION

The main conclusion that emerges from this work is that the marked variation observed in the amino acid sequence of flagellins in other bacteria (11) extends to *B. subtilis*. Strains such as W23 and BD71, for example, are genetically compatible, yet they show enormous differences in their peptide pattern; there are at least 10 peptide changes between these two strains. These differences are clearly reflected in the antigenic specificity of the flagellar filament and can be used to classify strains of *B. subtilis* according to serotype.

Antisera prepared against the filaments can be used for typing. These sera react with the filament but do not react extensively with the subunit. Among the 18 strains that were tested, they could be used to establish 5 clearly separable serological classes and there are undoubtedly more. Members of the same class show measurable cross-reaction with a given serum and appear to possess few if any antigenic determinants in common with members of other classes. Thus, for example, no cross-reaction could be detected between anti-BD71 and W23 flagella and, reciprocally, there was no cross-reaction between anti-W23 and BD71 flagella.

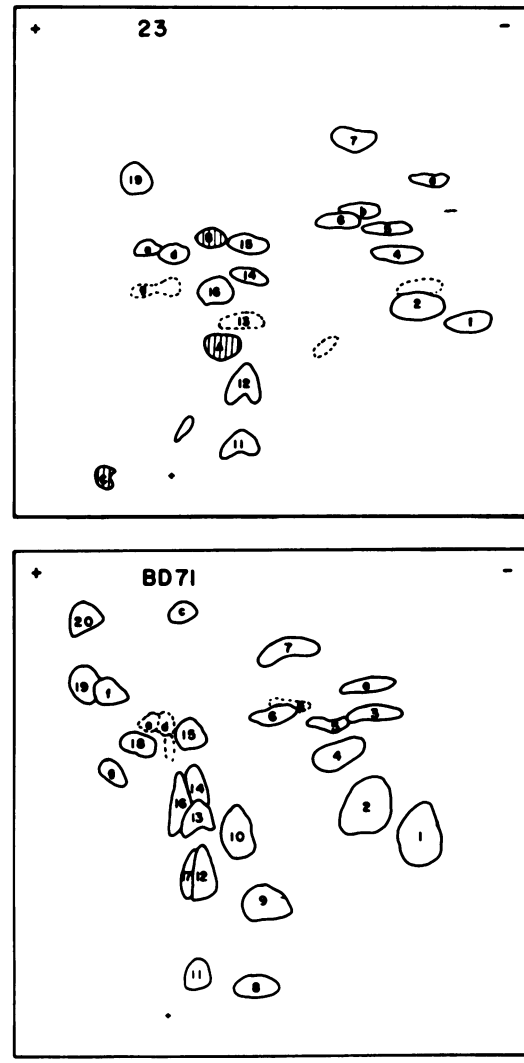

FIG. 3. *Peptide maps of BD71 and W23 flagellin. The origin is indicated by a + in the left-hand bottom corner. Peptides designated by arabic numerals are darkly staining peptides which are always clearly present. Peptides of variable staining intensity are identified by lower-case letters. Capital letters represent peptides not found in BD71.*

In previous work in other laboratories, antisubunit sera were prepared in two ways, first by direct intravenous injection of flagellin (9) and second by injecting periodate-treated flagellin (10). The antisera that resulted from both of these procedures contained high titers of antiflagellar filament antibody in addition to antisubunit antibody. We attempted a third distinct approach. Freshly prepared flagellin was bound

to methylated albumin and the complex was injected. One of the resulting antisera was found to contain high titers of antisubunit antibody and did not react with flagellar filaments. It is possible that, on binding to methylated albumin, the flagellin is spread out and denatured; thus previously inaccessible determinants become available to the immunogenic system. This serum was specific for flagellin derived from *B. subtilis* strains. We could not detect any cross-reaction, for example, with flagellin from *Escherichia coli*. It could, furthermore, clearly distinguish between different classes of *B. subtilis* flagella (Table 2). For example, the difference between the BD71 and the ATCC 13542 strains (index of dissimilarity of 2.4) is comparable to the serological differences found in the tryptophan synthetase α subunit between *E. coli* and *Salmonella typhimurium* (12). However, the antifilament serum was even more specific. We can speculate that the differences between these sera resulted from the antifilament antibodies being directed against regions of the protein that were unique to each class of flagellin and were phylogenetically very variable, whereas the antiflagellin antibody measured antigenic determinants that were relatively homologous and conserved.

The analysis of the tryptic peptides derived from the different classes of flagellin could be consistent with this interpretation. Proteins of the same class differed in only one or two peptides, whereas proteins of different classes contained extensive differences which could account for their unique antigenic reactivity with the antifilament serum. On the other hand, 9 of the 20 major peptides were present in all of the flagellins examined, and they may have been in part responsible for the similarities detected by the antiflagellin antibody. Furthermore, since antigenic homology is demonstrable only with the dissociated flagellin subunits, it is possible that the tryptic peptides common to all the strains represent regions that are not accessible to the antibody while the subunit is contained in the filament form, e.g., intersubunit binding sites or sites involved in maintaining the conformation of the subunit.

It clearly becomes difficult to try to interpret strictly both the serological data and the tryptic maps, because we do not know anything about the relative conformation of the various flagellins. Antigenicity derives from both the sequence and the tertiary structure of the molecule. Specific single amino acid replacements can markedly change the entire antigenic pattern of the flagella (7; S. Yamaguchi and T. Iino, Rep. Nat. Inst. Genet., Misima **16**:94) presumably by changing the configuration. On the other hand, many other single replacements may be tolerated with only very small changes in antigenicity. In some kinds of comparisons, the problem of conformational change can be minimized. For example, Yamaguchi and Iino (16) were able to do

TABLE 3. *Analysis of tryptic peptides*

Peptide[a]	Strains[b]						
	BD71	9524	12695	W23	13542	7067	9799
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	0	0	0	0	0
4	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+
8	+	+	0	0	0	0	+
9	+	+	0	0	0	**	0
10	+	+	0	0	0	0	+
11	+	+	+	+	0	+	+
12	+	+	+	+	0	+	+
13	+	+	0	0	+	**	+
14	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+
16	+	+	0	+	+	+	+
17	+	+	0	0	0	0	0
18	+	+	**	0	+	0	+
19	+	+	+	+	+	+	+
20	+	+	0	0	0	0	+
a	+	+	+	+	+	+	+
b	+	+	+	+	0	+	0
c	+	+	0	0	0	0	0
d	+	+	+	+	+	+	+
e	+	+	?	+	0	0	0
f	+	+	0	0	0	+	+
g	+	+	+	+	0	+	+
A	0	0	+	+	0	0	0
B	0	0	+	+	0	0	**
C	0	0	+	+	0	+	0
Additional	0	0	1	0	5	1	7

[a] Additional peptides not found in the prototype were determined by comparing 12695 and 7067 with W23 and 9524 and comparing 13542 and 9799 with BD71 (S. Emerson, Ph.D. Thesis, University of California, San Diego, 1970). The major flagellin peptides are identified by arabic numerals according to the scheme in Fig. 3. Lower-case letters represent peptides which are constantly present but are of variable staining intensity; upper-case letters represent three peptides which are constantly present but are of variable staining intensity; upper-case letter represent three peptides found in W23 but not in BD71. Peptides present in other flagellins but absent in W23 or BD71 are not numbered.

[b] +, Peptide present; 0, peptide absent; **, probably +, however, the flagellin was not co-run with that prototype.

genetic crosses between strains of *Salmonella* carrying different antigenic factors and obtain recombinant antigens. Upon analysis of the genetic, serological, and peptide pattern of these recombinants, it was found that they could be simply understood in terms of recombination of parental properties. However, this represents a relatively selective study, and a simple linear interpretation may not hold for all the antigenic factors. Clearly, extending the antigenic as well as the peptide map analysis to a larger number of *B. subtilis* strains will help to give a clearer picture of the relative complexity of the relationship between the antigenic specificity and the primary and secondary structure of flagellin.

ACKNOWLEDGMENT

This work was supported by a grant from the National Science Foundation (no. GB-15655).

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